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Chloroform Degradation in Methanogenic Methanol Enrichment Cultures and by *Methanosarcina barkeri* 227

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The effects of methanol addition and consumption on chloroform degradation rate and product distribution in methanogenic methanol enrichment cultures and in cultures of *Methanosarcina barkeri* 227 were investigated. Degradation of chloroform with initial concentrations up to 27.3 μM in enrichment cultures and 4.8 μM in pure cultures was stimulated by the addition of methanol. However, methanol consumption was inhibited by as little as 2.5 μM chloroform in enrichment cultures and 0.8 μM chloroform in pure cultures, suggesting that the presence of methanol, not its exact concentration or consumption rate, was the most significant variable affecting chloroform degradation rate. Methanol addition also significantly increased the number of moles of dichloromethane produced per mole of chloroform consumed. In enrichment cultures, the number of moles of dichloromethane produced per mole of chloroform consumed ranged from 0.7 (methanol consumption essentially uninhibited) to 0.35 (methanol consumption significantly inhibited) to less than 0.2 (methanol not added to the culture). In pure cultures, the number of moles of dichloromethane produced per mole of chloroform consumed was 0.47 when methanol was added and 0.24 when no methanol was added. Studies with [^{14}C]chloroform in both enrichment and pure cultures confirmed that methanol metabolism stimulated dichloromethane production compared with CO_2 production. The results indicate that while the addition of methanol significantly stimulated chloroform degradation in both methanogenic methanol enrichment cultures and cultures of *M. barkeri* 227, the prospects for use of methanol as a growth substrate for anaerobic chloroform-degrading systems may be limited unless the increased production of undesirable chloroform degradation products and the inhibition of methanol consumption can be mitigated.

Trichloromethane (chloroform) is a suspected human carcinogen (4) and a common groundwater contaminant (13, 15). Chloroform migrates relatively rapidly (23) and may move from highly contaminated groundwaters underneath leaking hazardous waste landfills and other improper storage facilities to contaminate potable waters. Chloroform-contaminated groundwaters should be remediated as near to the contamination source as possible.

Chloroform can be aerobically degraded by methanotrophic organisms (1, 21), ammonia-oxidizing organisms (26), and a recombinant pseudomonad expressing soluble methane monooxygenase (16). However, in methanotrophic cultures, chloroform and methane compete for the reaction site, soluble methane monooxygenase, decreasing the reaction rate of each (20, 21, 24). Furthermore, the requirement for dissolved oxygen and methane may impose practical and economical limitations on aerobic degradation.

Anaerobic systems may provide an alternative to aerobic systems for chloroform degradation. Chloroform can be degraded anaerobically to CO_2 and dichloromethane by methanogenic enrichment cultures and pure methanogenic cultures (3, 5, 11, 17, 19) and also by nonmethanogenic anaerobic cultures (6, 7, 10). Research has provided insight into possible chloroform degradation mechanisms by determining that cofactors found in methanogens and other anaerobic organisms, such as coenzyme F_{430} and cobalamins, are capable of catalyzing chloroform degradation in vitro in reduced systems (17, 18, 27). However, although chloroform degradation in methanogenic cultures could be stimulated by methanol addition (9, 19), chloroform remains extremely inhibitory to methanogenesis (2, 22, 28).

To design effective anaerobic remediation schemes for chloroform-containing waters, additional information is required. The sustainability of chloroform degradation must be examined, with the first step being to identify a stimulatory growth substrate that can be consumed in the presence of chloroform and to understand its stimulatory mechanism. The distribution of degradation products must also be confirmed, as well as the relationship of that distribution to consumption of a growth substrate. Chloroform degradation products such as dichloromethane are undesirable and, if formed, may require additional treatment steps for removal. In this paper, we examine chloroform degradation in both a methanogenic methanol enrichment culture and cultures of *Methanosarcina barkeri* 227. Relationships between chloroform degradation rate and methanol addition/consumption and between dichloromethane production and methanol consumption are identified.

MATERIALS AND METHODS

Chemicals. Chloroform, dichloromethane (99% pure; Supelco, Inc., Bellefonte, Pa.), and methane gas (99.0% pure; Scott Specialty Gases, South Plainfield, N.J.) were used for gas chromatograph calibration. Chloroform used to prepare the chloroform stock solution was obtained from Fisher Scientific, Pittsburg, Pa., and was 99.0% pure. Methanol (99.8%) and Difco yeast extract used to prepare growth substrate solutions were obtained from Fisher Scientific.

Alcohol oxidase (from *Pichia pastoris*, phosphate-buffered 60% sucrose solution), peroxidase (type I from horseradish, solid), and 2,2′-azino-di-(3-ethyl)-benzthiazoline-6-sulfonic acid (ABTS; 98%, diammonium salt) (all from Sigma Chemical Co., St. Louis, Mo.) were used to prepare the enzymatic reagent for methanol analysis. [^{14}C]chloroform (62 μCi/ml, >98% pure) was obtained from

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ICN Biomedicals, Irvine, Calif. ScintiVerse-E liquid scintillation cocktail (Fisher Scientific) was used for radioactivity measurements.

Experimental operation. The inoculum source for methanol enrichment culture experiments was a stirred, 7.5-liter, semicontinuous enrichment reactor operating with a 15-day retention time and kept at 34 to 35°C in a constant-temperature room. The reactor was seeded with 2.5 liters from a 15-liter, stirred semicontinuous digester initially charged in 1986 that received 10 g of chemical oxygen demand per liter of complex feed and operated with a 20-day retention time (8). Once each day, gas production was measured, 500 ml of culture was removed, 5 ml of concentrated methanol solution (333.3 g of methanol and 5 g of yeast extract per liter) was added, and 495 ml of sulfide-reduced, bicarbonate-buffered basal medium (containing, per liter of distilled water, $NaHCO_3$, 6.0 g; $Na_2S \cdot 9H_2O$, 0.5 g; NH_4Cl, 0.285 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $K_2HPO_4 \cdot 3H_2O$, 0.1 g; $FeCl_2 \cdot 4H_2O$, 0.1 g; KH_2PO_4, 0.055 g; resazurin, 1 mg; and trace metal solution, 5 ml [containing, per liter of distilled water, $MnCl_2 \cdot 4H_2O$, 0.2 g; $CoCl_2 \cdot 6H_2O$, 0.34 g; $ZnCl_2$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; H_3BO_3, 0.038 g; $NiCl_2 \cdot 6H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.04 g; NH_4VO_3, 0.04 g; KI, 0.8 g; and cysteine HCl, 0.8 g]) was added to return the liquid volume to 7.5 liters. Microscopic examination of the enrichment reactor contents showed the prevailing morphology to be very large, sarcina-like clusters. The cultures were not pure; highly motile rods, although present in small numbers, were also evident.

Batch experiments were conducted in 160-ml serum bottles capped with Teflon-lined rubber or butyl rubber septa held in place with aluminum crimp seals. Methanol enrichment culture (100 ml) was dispensed from the reactor with an anaerobic buret apparatus kept under a 75% N_2–25% CO_2 headspace. Trace oxygen was removed from the gas stream by washing in a titanium(III) citrate solution (8). To avoid negative pressure inside a bottle as a result of liquid and headspace sampling, 12 ml of 75% N_2–25% CO_2 was added to the bottle with a gas-tight syringe immediately after capping.

M. barkeri 227 cultures were grown in medium containing (per liter of distilled water) NH_4Cl, 0.5 g; K_2HPO_4, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $MgCl_2$, 0.1 g; trace metal solution, 10 ml (containing, per liter, $FeSO_4 \cdot 7H_2O$, 0.556 g; $MnSO_4 \cdot H_2O$, 0.086 g; $CoCl_2 \cdot 6H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.21 g; H_3BO_3, 0.019 g; nitrilotriacetic acid, 4.5 g; $NiCl_2$, 0.02 g; and $Na_2MoO_4 \cdot 2H_2O$, 0.01 g; the pH was adjusted to 7.0 with 10 N KOH); and 0.1% resazurin solution, 1 ml. The medium was gassed with N_2 for 45 min, and 100 ml was dispensed into 160-ml serum bottles in an anaerobic glove box (29). The bottles were capped with Teflon-lined butyl rubber septa and autoclaved. Bottle headspaces were then replaced with 70% N_2–30% CO_2, and the following sterile, anoxic stock solutions were added: 1.0 ml of 10% $NaHCO_3$, 0.5 ml of 5% $Na_2S \cdot 9H_2O$, 0.2 ml of vitamin solution (containing, per liter, biotin, 20 mg; folic acid, 20 mg; pyridoxine HCl, 100 mg; thiamine HCl, 50 mg; riboflavin, 50 mg; nicotinic acid, 50 mg; DL-calcium pantothenate, 50 mg; vitamin B_{12}, 1 mg; *p*-aminobenzoic acid, 50 mg; and lipoic acid, 50 mg), and 2.0 ml of 10% methanol solution.

The bottles were then inoculated with 2.0 ml of *M. barkeri* 227 culture (provided by Stephen Zinder, Cornell University). They were incubated at 37°C for 4 days, another 1.0 ml of 10% methanol solution was added, and they were incubated at 37°C for another 2 days prior to use in experiments.

Chloroform was added to inoculated bottles from a chloroform-saturated water stock. The quantity of chloroform determined by headspace analysis after 5 to 10 minutes of equilibration in a 35°C orbital shaker bath was defined as the initial quantity. The initial aqueous chloroform concentration (micromolar; calculated by considering the distribution of chloroform between gas and liquid phases) was also determined. Methanol was added as a 20% aqueous solution. All cultures were continuously agitated in a 35°C orbital shaker bath.

Measurements. Chloroform, dichloromethane, and methane were measured by gas-chromatographic analysis of headspace samples. A Perkin-Elmer model 8500 gas chromatograph outfitted with a flame ionization detector and a stainless steel column (3.2 mm by 2.44 m) packed with 1% SP-1000 on 60/80 Carbopack B (Supelco, Inc.) was used. Injector and detector temperatures were 200 and 250°C, respectively. Nitrogen carrier gas flow was 30 ml/min. The oven temperature program started at 100°C for 1.0 min and then increased at 20°C/min to 150°C, where it was held for 2.5 min.

Calibration factors were determined by the method of Gossett (12) and adjusted for changes in liquid and headspace volume due to liquid sampling by using Henry's law. The Henry's law constants used (at 35°C) were 0.227, 0.129, and 29.5 mol/liter (gas) per mol/liter (liquid) for chloroform, dichloromethane, and methane, respectively (12).

Methanol concentrations were determined enzymatically with a Beckman model 3600 spectrophotometer (14). Volatile suspended solids (VSS) measurements (1a) were conducted in triplicate for each bottle at the completion of each experiment.

Recovery of [^{14}C]compounds. To confirm that the ^{14}C label was on chloroform, 100 μl of an aqueous stock solution containing 6.8 μCi/ml was added to a 160-ml serum bottle containing 100 ml of distilled water and equilibrated at 35°C. Only 1% of the label recovered from fractionation of a headspace sample was not trapped during the chloroform interval (recovery was 88.5%). Additionally, the Henry's law constant of the labeled constituent, determined from counting unfractionated headspace and liquid samples, was calculated to be 0.225, very near the 0.227 value for chloroform (12). ^{14}C activity was determined by counting on a Beckman 9800 liquid scintillation counter.

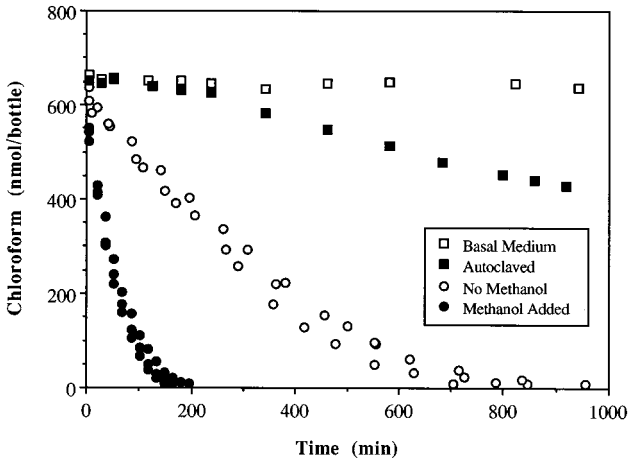

FIG. 1. Chloroform removal in basal medium, autoclaved methanol enrichment culture, methanol enrichment culture with no methanol added (triplicate), and methanol enrichment culture with methanol added (triplicate). Biomass = 408 ± 12 mg of VSS per liter.

Experimental bottles each received 100 μl of the ^{14}C-labeled aqueous stock (6.8 μCi/ml). Unlabeled, chloroform-saturated water was added to bring the total chloroform concentration to the desired level. Volatile ^{14}C-labeled compounds were recovered by the gas-chromatographic combustion technique described previously (8). After the addition of 2.0 ml of 5 N NaOH to experimental bottles to trap CO_2 in solution, headspace samples were fractionated on a gas chromatograph. Chloroform, dichloromethane, and chloromethane were separated on the 60/80 Carbopack column, with the oven temperature held at 60°C for 1.5 min and then increased at 20.0°C/min to 150°C, where it was held for 2.0 min. Methane and CO were separated on a 100/120 Carbosieve S-II column (3.2 mm by 3.2 m; Supelco, Inc.) with the oven temperature held at 150°C for 11 min. Trapping times were determined as described previously (8). The dimensionless Henry's law constants used (at 35°C) were 0.499 for chloromethane (12) and 48.7 for CO (25).

The ^{14}C label remaining as CO_2 was determined by stripping a 20.0-ml aliquot with nitrogen into a 0.5 N NaOH trap (8). The presence of $^{14}CO_2$ in the NaOH trap was confirmed by precipitation with $Ba(OH)_2$ and filtration through a 0.45-μm-pore-size membrane filter. The ^{14}C label remaining as nonstrippable residue (NSR) was separated into soluble and insoluble fractions by filtration through a 0.45-μm-pore-size membrane filter.

RESULTS

Chloroform degradation in methanol enrichment culture. Chloroform was removed, without lag, in methanol enrichment cultures (Fig. 1). Cultures that received methanol (5.6 mM) degraded chloroform more rapidly than did those without methanol. The addition of methanol also stimulated chloroform removal over a wide range of chloroform concentrations (Fig. 2A), although methanol consumption rates were inhibited by chloroform (Fig. 2B). Methane production was stoichiometric with methanol consumption (data not shown).

The chloroform degradation rates in cultures receiving no methanol were a function of both initial and remaining chloroform concentrations. Figure 2A shows that the slope of the chloroform degradation curve (chloroform degradation rate) at any remaining chloroform concentration was steeper in cultures that received a greater initial chloroform concentration. Chloroform degradation rates in cultures receiving methanol showed a different relationship to chloroform concentration. As Fig. 2A shows, the chloroform degradation rates at any remaining chloroform concentration were approximately equal, suggesting that the initial chloroform concentration did not affect the instantaneous chloroform degradation rate. The maximum chloroform degradation rates in bottles receiving methanol, estimated as the slopes of the linear portions of the degradation curves, averaged 7.1 ± 0.4 nmol per bottle per min

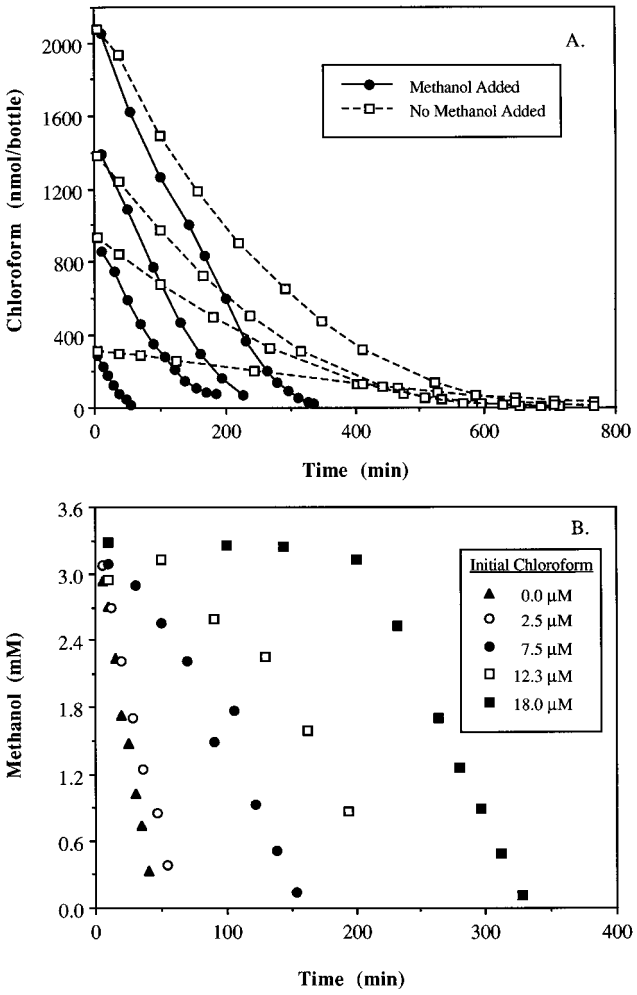

FIG. 2. (A) Chloroform removal in methanol enrichment cultures with and without methanol addition. (B) Methanol consumption in methanol enrichment cultures receiving chloroform (initial aqueous chloroform concentrations noted). Biomass = 405 ± 11 mg of VSS per liter.

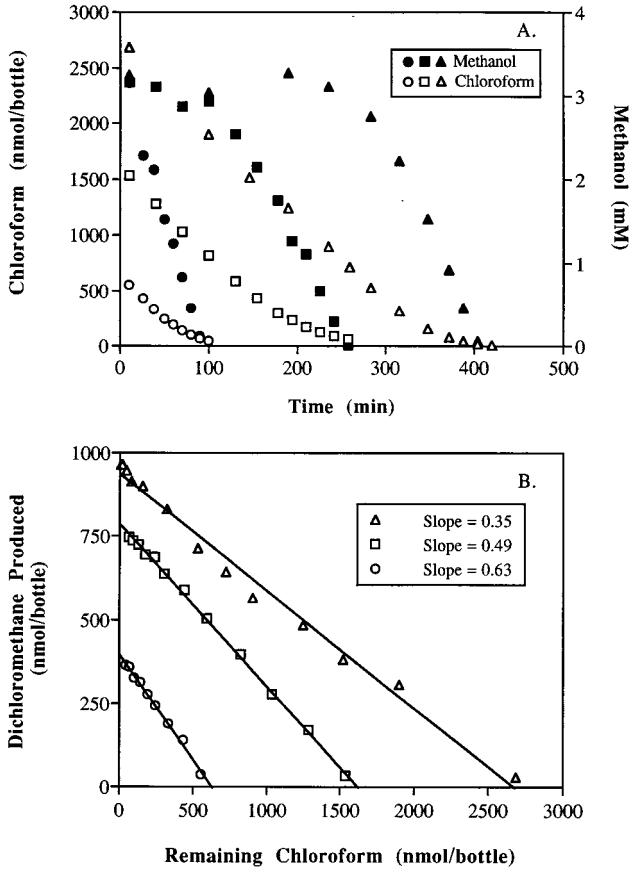

FIG. 3. (A) Chloroform and methanol removal in three representative methanol enrichment cultures (open and solid symbols of the same shape are results from one bottle). (B) Dichloromethane production in three representative methanol enrichment cultures (lines represent best-fit curves from linear regression). Biomass = 392 ± 16 mg of VSS per liter.

(slope ± standard deviation), further indicating that the initial chloroform concentration did not affect the instantaneous chloroform degradation rate.

The addition of methanol stimulated chloroform degradation, but the chloroform degradation rate was a function of neither instantaneous methanol concentration (at least at the concentrations examined) nor methanol consumption rate. Although chloroform significantly inhibited methanol consumption (Fig. 2B), the maximum chloroform degradation rates in bottles receiving methanol were essentially constant, even when methanol consumption was undetectable for over 200 min. These results suggest that the presence of methanol, not its concentration or consumption rate, was the most significant variable affecting the chloroform degradation rate.

Methanol consumption rates were a nonlinear function of the remaining chloroform concentration (Fig. 2B). As the initial chloroform concentration was increased, the time during which methanol consumption was not detectable also increased, even though chloroform degradation occurred. After sufficient chloroform was degraded, methanol consumption rates became detectable and increased to a maximum value of 21 ± 5 μM/min, well below the consumption rate of 76 ± 2 μM/min observed when chloroform was not added. As chloroform degradation continued, methanol consumption rates did not return to the uninhibited rate, suggesting that the initial concentration of chloroform to which the culture was exposed irreversibly removed a fraction of the methanol-consuming capability of the culture.

Effect of methanol on product distribution. The products of chloroform degradation in methanogenic cultures have been previously determined by several researchers (3, 5, 11, 17, 19). However, the product distribution as a function of methanol consumption was not reported. To examine the relationship of product distribution to methanol addition and initial chloroform concentration, 18 bottles inoculated with methanol enrichment culture were examined. Thirteen bottles received methanol (3.1 mM) and different levels of chloroform from 88 to 3,110 nmol per bottle (initial aqueous chloroform concentration, up to 27.3 μM), and five bottles received chloroform only, up to 2,080 nmol per bottle (18.3 μM). Chloroform removal and methanol consumption were as expected (Fig. 3A).

To determine the number of moles of dichloromethane produced per mole of chloroform consumed (the molar ratio of dichloromethane produced), the quantity of dichloromethane (nanomoles per bottle) at any time was plotted against the quantity of chloroform remaining (nanomoles per bottle) at that time. These plots for the bottles receiving no methanol

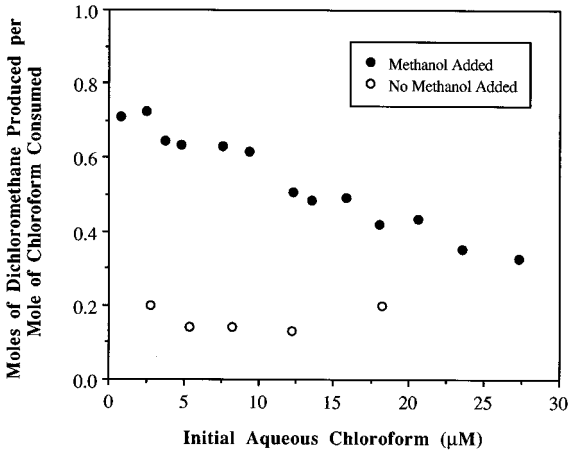

FIG. 4. Average dichloromethane production from chloroform in methanol enrichment cultures with and without methanol addition. Biomass = 404 ± 20 mg of VSS per liter.

exhibited some curvature (data not shown), indicating that instantaneous dichloromethane production varied with the remaining chloroform concentration.

The plots of dichloromethane produced versus remaining chloroform for the bottles receiving methanol were linear (Fig. 3B). Dichloromethane production was not a function of the remaining chloroform concentration or methanol consumption rate, both of which varied significantly over time (compare Fig. 3A and B). However, the average molar ratio of dichloromethane produced (estimated as the slope of the best-fit line obtained from linear regression) decreased as the initial chloroform concentration increased (Fig. 3B and 4).

The addition of methanol significantly increased dichloromethane production (Fig. 4), even though the molar ratio of dichloromethane produced was not related to the time-varying methanol consumption rate (Fig. 3A and B). Instead, dichloromethane production increased with increasing initial methanol consumption rate, although the relationship was nonlinear (Fig. 5). In cultures receiving methanol, the molar ratio of dichloromethane produced ranged from over 0.7 for initial methanol consumption rates greater than 50 μM/min to less than 0.35 for initial methanol consumption rates less than 1 μM/min. The molar ratio of dichloromethane produced in the absence of methanol addition ranged from 0.13 to 0.20, suggesting that even at immeasurable methanol consumption rates, the metabolism of methanol by the culture affected the product distribution.

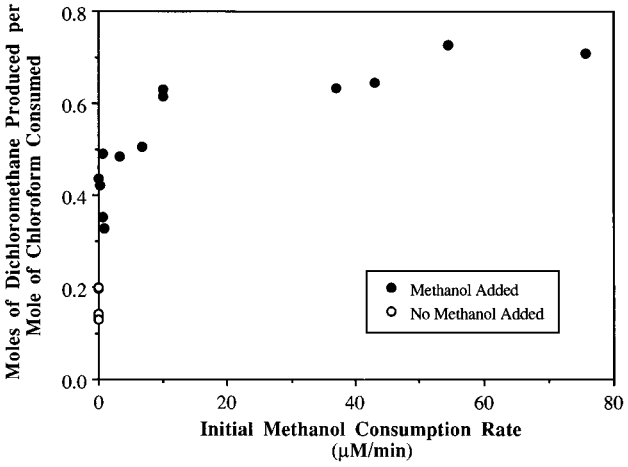

FIG. 5. Average dichloromethane production from chloroform in methanol enrichment cultures as a function of initial methanol consumption rate. Biomass = 404 ± 20 mg of VSS per liter.

The effect of methanol addition on product distribution was further investigated with [^{14}C]chloroform (Table 1). As expected, the primary products in all bottles were dichloromethane and CO_2, with most of the remaining label detected in the NSR. The identities of the compounds in the NSR were not determined.

To compare the distribution of reduced and oxidized products, the ratios of dichloromethane recovered to CO_2 recovered were calculated. At the lower initial chloroform concentrations, at which inhibition of the initial methanol consumption rate is expected to be slight, the addition of methanol apparently shifted the product distribution to dichloromethane (Table 1), qualitatively supporting the results with unlabeled chloroform. At the high initial chloroform concentrations where significant inhibition of the initial methanol consumption rate is expected, the addition of methanol apparently had little effect on the product distribution, as expected from the results with unlabeled chloroform.

Chloroform degradation in *M. barkeri* 227. Chloroform was removed, without lag, in cultures of *M. barkeri* 227 (Fig. 6A). As was observed in the enrichment culture, methanol addition stimulated chloroform removal. Additionally, chloroform degradation rates in the absence of methanol were a function of both initial and remaining chloroform concentrations, while the instantaneous chloroform degradation rates in the presence of methanol were similar at any remaining chloroform concentration, regardless of the initial chloroform concentration. Furthermore, as was observed in the enrichment culture, the instantaneous chloroform degradation rate did not appear to be a function of either remaining methanol concentration or methanol consumption rate, both of which changed significantly over time (Fig. 6B).

Methanol consumption was clearly inhibited by chloroform, although it was observed in the presence of chloroform (Fig. 6B), with stoichiometric methane production (data not shown). Additionally, as was observed in the enrichment cultures, methanol consumption rates increased as remaining chloroform concentrations decreased, although not to uninhibited rates.

The molar ratios of dichloromethane produced from chloroform were calculated from plots of the quantity of dichloromethane produced versus the quantity of chloroform remaining at that time (Table 2). As observed in the enrichment culture, the addition of methanol significantly increased (99% confidence interval) dichloromethane production. Additionally, dichloromethane production in the cultures receiving no methanol did not significantly increase with increasing initial chloroform concentration. Unlike the enrichment culture results, however, dichloromethane production in cultures receiving methanol did not appear to be related to initial chloroform concentration.

The distributions of [^{14}C]chloroform degradation products in *M. barkeri* 227 cultures are shown in Table 3. The primary products were dichloromethane and CO_2, with most of the remaining label detected in the NSR, particularly the soluble fraction of the NSR. The ^{14}C distribution results qualitatively support the results obtained with unlabeled chloroform, with the addition of methanol causing a shift to dichloromethane production.

TABLE 1. Distribution of products recovered from [^{14}C]chloroform addition to methanol enrichment culture[a]

Product	% of recovered product[b] at following initial concns[c]:			
	4.6 μM $CHCl_3$ + 0 mM CH_3OH (112.4%)	4.2 μM $CHCl_3$ + 3.1 mM CH_3OH (104.9%)	21.3 μM $CHCl_3$ + 0 mM CH_3OH (101.8%)	20.6 μM $CHCl_3$ + 6.3 mM CH_3OH (92.3%)
CH_3Cl	1.6	0.0	0.0	0.0
CH_2Cl_2	48.0	69.6	46.4	49.4
$CHCl_3$	0.0	2.8	0.0	0.0
CO fraction	0.0	0.0	0.0	0.0
CH_4	0.9	0.8	0.1	0.5
CO_2	44.0	15.9	46.5	41.9
Insoluble NSR	3.7	6.0	4.0	5.1
Soluble NSR	1.8	4.9	3.0	3.1
CH_2Cl_2/CO_2 recovery ratio	1.09	4.38	1.00	1.18

[a] Biomass, 384 ± 11 mg of VSS per liter.
[b] Versus addition of 0.71 μCi to the first two bottles and 0.72 μCi to the second two bottles. Determined by adding 100 μl of labeled stock to the cocktail and counting.
[c] Aqueous concentrations of chloroform are given.

DISCUSSION

Methanol addition stimulated chloroform degradation in both methanogenic methanol enrichment cultures and *M. barkeri* 227 cultures (Fig. 1, 2A, 3A, and 6A). This is in contrast to aerobic chloroform degradation, in which the growth substrate competes with chloroform for the reaction site (20, 21, 24). Nevertheless, the stimulation of chloroform degradation by methanol addition under methanogenic conditions was not unexpected. Previous researchers identified this phenomenon in mixed methanogenic cultures (9) and cultures of *Methanosarcina* sp. strain DCM and *M. mazei* S6 (19). In those investigations, however, methanol concentrations were not measured, so the relationships of chloroform degradation rate and product distribution to methanol concentration and methanol consumption rate could not be determined.

Although methanol addition clearly stimulated chloroform degradation, there was no indication that remaining methanol concentration or methanol consumption rate affected the instantaneous chloroform degradation rate in either the methanol enrichment or *M. barkeri* 227 cultures. This apparent lack of relationship is particularly surprising when the methanol consumption results are considered. The maximum chloroform degradation rates in the enrichment cultures were essentially constant when methanol was present, even though the initial methanol consumption rates varied by almost 2 orders of magnitude. *M. barkeri* 227 cultures showed qualitatively similar results, suggesting that the phenomenon observed in the enrichment cultures could be due in part to methanogenic organisms. These observations suggest that in an anaerobic treatment system designed to remove chloroform, very little methanol consumption would be required to stimulate chloroform degradation.

That methanol consumption rates did not return to uninhibited rates as chloroform concentrations decreased suggests that some fraction of the methanol metabolic pathway in the organisms was irreversibly inhibited. It is possible that the extent of inhibition was related to the initial chloroform concentration. This inhibition of methanol consumption did not appear to affect chloroform degradation rates in short-term bottle experiments. However, the long-term sustainability of chloroform degradation in anaerobic chloroform treatment systems is questionable if organism growth cannot occur. Additional research investigating inhibition mechanisms should be conducted, in particular to examine this sustainability question.

TABLE 2. Molar ratio of dichloromethane produced per mole of chloroform consumed for *M. barkeri* 227 cultures with and without methanol addition[a]

Initial chloroform concn (μM)[b]	Initial methanol concn (mM)	Amt of dichloromethane produced/mol of chloroform consumed (nmol/nmol)[c]
0.7	4.4	0.39[d]
2.2	7.8	0.61
3.8	4.7	0.42
4.4	6.3	0.44
4.9	6.3	0.47
Mean (SD)		0.47 (0.09)
0.8	0	0.18[d]
2.1	0	0.25
3.8	0	0.25
4.6	0	0.25
4.9	0	0.26
Mean (SD)		0.24 (0.03)

[a] Biomass = 231 ± 10 mg of VSS per liter.
[b] Aqueous concentration.
[c] Determined by linear regression of dichloromethane produced versus remaining chloroform quantity unless otherwise noted.
[d] Determined as the ratio of the final dichloromethane quantity divided by the initial chloroform quantity.

TABLE 3. Distribution of products recovered from [^{14}C]chloroform addition to *M. barkeri* 227 culture[a]

Product	% of recovered product[b] at following concns[c]:	
	3.1 μM $CHCl_3$ + 0 mM CH_3OH (99.7%)	2.9 μM $CHCl_3$ + 6.3 mM CH_3OH (102.9%)
CH_3Cl	0.7	1.5
CH_2Cl_2	29.9	56.2
$CHCl_3$	0.0	0.0
CO fraction	0.0	0.0
CH_4	0.6	1.6
CO_2	53.4	19.2
Insoluble NSR	2.9	4.3
Soluble NSR	12.5	17.2
CH_2Cl_2/CO_2 recovery ratio	0.56	2.93

[a] Average biomass = 229 mg of VSS per liter.
[b] Versus added quantities of 0.61 μCi determined by adding 100 μl of labeled stock to the cocktail and counting. Values in parentheses represent the overall percent recovery for the bottle.
[c] Aqueous concentrations of chloroform are given.

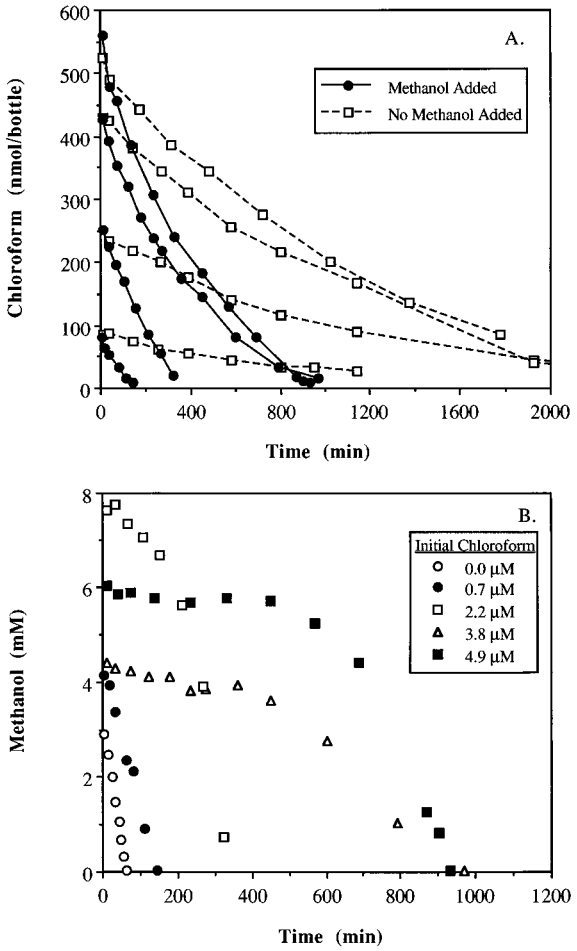

FIG. 6. (A) Chloroform removal in *M. barkeri* 227 cultures with and without methanol addition (duplicates at the highest chloroform concentration not shown for clarity). (B) Methanol consumption in *M. barkeri* 227 cultures receiving chloroform (initial aqueous chloroform concentrations noted). Biomass = 231 ± 10 mg of VSS per liter.

In both enrichment and *M. barkeri* 227 cultures, methanol addition increased the molar ratio of dichloromethane produced from chloroform degradation. In enrichment cultures, dichloromethane production appeared to be a function of initial chloroform concentration (in cultures receiving methanol) and/or initial methanol consumption rate. In *M. barkeri* 227 cultures, there was no apparent relationship with either of these variables, perhaps suggesting that the relationship of dichloromethane production to these variables was an artifact of the enrichment culture. However, in both enrichment and pure cultures, dichloromethane production was not a function of remaining chloroform concentration, instantaneous chloroform degradation rate, remaining methanol concentration, or instantaneous methanol consumption rate.

The mechanism by which methanol metabolism stimulated chloroform degradation was not determined. However, one hypothesis can be proposed by assuming that chloroform rapidly binds to a reaction site, perhaps a cobalamin or coenzyme F_{430} (17, 18, 27). The rate of chloroform carbon removal from the reaction site would be a function of other reactants available near the site and would control the overall chloroform degradation rate. Methanol metabolism would increase the concentration of constituents in the cell that are capable of supplying electrons to reduce the bound chloroform to dichloromethane.

This hypothesis predicts that chloroform degradation will occur in organisms both receiving and not receiving substrate, although in the absence of substrate addition, the capability of the organisms to degrade chloroform may be limited by the initial pool of available reactants. Additionally, this hypothesis predicts that substrate metabolism, in this case methanol, would increase the chloroform degradation rate compared with that in cells not receiving substrate. Furthermore, methanol metabolism should increase the molar ratio of dichloromethane produced from chloroform. These predictions were observed in methanogenic methanol enrichment cultures (Table 1; Fig. 2A, 3A, and 5) and *M. barkeri* 227 cultures (Tables 2 and 3; Fig. 6A). Cultures that received no methanol would also be expected to produce dichloromethane to the extent that electron-donating constituents are available. This was observed in both enrichment and pure cultures.

With two further assumptions, this hypothesis also predicts the significant inhibition of methanol consumption caused by chloroform addition and the observation that chloroform degradation was stimulated in the presence of methanol even when methanol consumption was not measurable. The first assumption requires that at least one in vivo reaction site for chloroform also be an integral part of the methanol metabolic pathway. Several possible reaction sites may be considered, because the assumption does not require that there be only one in vivo reaction site. This assumption allows the hypothesis to explain the observed inhibition of methanol consumption. The second assumption requires that the activity of the reaction site(s) found in the methanol metabolic pathway be increased by the addition of methanol. If this assumption is valid, the presence of methanol above a certain activation concentration would increase the chloroform degradation rate by increasing the availability of active reaction sites, even though the methanol consumption rate remains immeasurably low.

Additional research should be conducted to identify the in vivo chloroform reaction sites and their relationship to methanol metabolism pathways. Determination of the soluble and insoluble NSR observed with [^{14}C]chloroform may be a logical step toward accomplishing that identification.

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